Pollution-induced community tolerance of freshwater biofilms: Measuring heterotrophic tolerance to Pb using an enzymatic toxicity test

L.C. Fechner, M. Dufour, C. Gourlay-Francé

► To cite this version:

L.C. Fechner, M. Dufour, C. Gourlay-Francé. Pollution-induced community tolerance of freshwater biofilms: Measuring heterotrophic tolerance to Pb using an enzymatic toxicity test. *Ecotoxicology*, 2012, 21 (8), p. 2123 - p. 2131. hal-00764058

HAL Id: hal-00764058

<https://hal.science/hal-00764058>

Submitted on 12 Dec 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Pollution-Induced Community Tolerance of freshwater biofilms:
measuring heterotrophic tolerance to Pb using an enzymatic toxicity test**

Ecotoxicology, 21(8):2123–2131

Accepted: 7 June 2012 / Published online: 24 June 2012

The original publication is available at <http://www.springer.com>

Lise C. Fechner^{123^}, Marine Perret¹³, Catherine Gourlay-Francé¹³

¹Irstea Antony – Unité de Recherche Hydrosystèmes et Bioprocédés (HBAN)

1 rue Pierre-Gilles de Gennes, CS 10030 F-92761 Antony Cedex, France

²AgroParisTech, 19 avenue du Maine, 75732, Paris, France

³FIRE

FR-3020, 4 Place Jussieu 75005, Paris, France

[^]Tel: +33140966568

[^]Fax: +33140966199

[^]E-mail: lise.fechner@irstea.fr

Abstract

This study aims at investigating the impacts of Pb on freshwater biofilms with a Pollution-Induced Community Tolerance (PICT) approach using a recently developed short-term toxicity test based on β -glucosidase activity to measure biofilms' tolerance to Pb. We first investigated more closely the influence of the TSS (Total Suspended Solids) concentrations of the biofilm suspensions used for short-term toxicity tests performed to assess Pb tolerance. The Pb EC₅₀ values of four dilutions of the same biofilm suspension increased with their TSS concentrations. TSS-normalization allowed to obtain a unique measure of Pb tolerance, thus confirming that TSS-normalization of EC₅₀ values is a good means to estimate biofilm tolerance to Pb. The experiment was repeated with three different biofilm samples collected at different time and date. Second, biofilms were exposed to Pb (0, 1, 10 and 100 μ g/L) for three weeks in microcosms to assess the impacts of Pb exposure on the communities. An increase in Pb tolerance was observed for the biofilm exposed to 100 μ g/L. Automated Ribosomal Intergenic Spacer Analysis revealed modifications of bacterial and eukaryotic community structure with Pb exposure. Moreover, exposure to 100 μ g/L Pb also led to an increase in Zn tolerance but not Cu tolerance. This study shows that tolerance acquisition to Pb can be detected after exposure to environmental concentrations of Pb using a PICT methodology and normalized EC₅₀ values as measures of Pb tolerance.

Key words: biofilm, metal tolerance, lead, toxicity, PICT, co-tolerance

Introduction

Aquatic systems in urban areas are subjected to chronic and diffuse multi-contamination by mixtures of micropollutants. Those substances are present in river waters at low concentrations which do not always lead to clear biological effects. Being able to assess the impacts of such low contamination levels on aquatic systems is one of the challenges of aquatic ecotoxicology. To address the issue of linking chemical contamination to biological impairment, the Pollution-Induced Community Tolerance (PICT) approach was proposed by Blanck et al. (2002). PICT uses tolerance acquisition at the community level as a biological response to exposure to a toxic agent. An increase in community tolerance to a toxic agent is assumed to be related to the pressure exerted by the toxic agent leading to selection (of tolerant species) or adaptation (both genotypic and phenotypic) processes within the exposed community. Determination of community tolerance is achieved by the means of short-term toxicity tests based on physiological activities (for instance respiration, photosynthesis, etc.) and community tolerance is usually expressed as the EC_{50} value derived from such a short-term test. Comparisons of EC_{50} values obtained from different communities allow to discriminate which community has been exposed to the toxic agent under study or not: an EC_{50} value higher than the EC_{50} value of a control community indicates a PICT effect. The PICT approach has been used on various algal communities exposed to herbicides (for instance Pesce et al., 2010) or on freshwater biofilms, for instance exposed to metals (Fechner et al., 2012a).

Biofilms (or periphyton) are complex communities of both autotrophic and heterotrophic microorganisms (algae, bacteria, fungi) that develop on submerged surfaces like rocks, macrophytes or sediments. Biofilms play an important ecological role in aquatic systems both as primary producers (autotrophic microorganisms) and as recyclers of organic matter

(heterotrophic microorganisms). They also respond fast to any stress and are thus usually considered as good early-warning indicators of the overall health of aquatic systems (Sabater *et al.*, 2002). Biofilms have been used to identify PICT effects with various herbicides, antifouling agents or metals (for a review see Blanck, 2002; Sabater *et al.*, 2007) .

In our previous study, a short-term toxicity test based on β -glucosidase activity (β -glucosidase toxicity test) was used to measure metal tolerance levels of heterotrophic communities from freshwater biofilms collected in an urban area (Fechner *et al.*, 2010a) . Biofilms were collected on artificial substrates immersed in the river for several weeks and then scraped to make biofilm suspensions in mineral water. Short-term toxicity tests were performed on those biofilm suspensions. To obtain a reliable measure of community tolerance, a normalized EC_{50} value was proposed in order to take into account modifications of metal bioavailability during the short-term test. Indeed, the issue of bioavailability in the test medium is rarely addressed although modifications of metal bioavailability, for instance related to biofilm composition (Extracellular Polymeric Substances or EPS from the biofilm matrix, organic or mineral particles from the river, etc.), might change any toxicity data derived from the tests. In particular, we observed that EC_{50} values varied with the TSS (Total Suspended Solids) concentration of the biofilm suspensions used for metal toxicity testing. TSS-normalization of EC_{50} values thus appeared as a reliable means to estimate metal tolerance (Fechner *et al.*, 2010a) for metals like Cu, Cd, Ni and Zn. Yet, we concluded that normalization of EC_{50} values might not necessarily be an efficient means to reliably assess Pb tolerance of freshwater biofilms and that further investigations ought to be conducted.

Pb has been used for various human activities for centuries and is one of the priority substances of the Water Framework Directive. Several studies suggest that metal bioavailability and toxicity

in the presence of natural organic matter is likely to be affected differently in the case of Pb than in the case of other metals like Cu or Zn (for instance Tsiridis et al., 2006; Lamelas et al., 2009; Sánchez-Marín et al., 2010). Indeed, the presence of natural organic matter has been shown to either enhance or decrease Pb toxicity towards aquatic organisms depending on the studies (Sánchez-Marín *et al.*, 2010). Therefore, being able to reliably estimate biofilm tolerance to Pb is a crucial issue in order to assess the impacts of urban metallic exposure on freshwater biofilms.

The aim of this study was first to investigate the reliability of Pb tolerance measurements using a PICT approach and the β -glucosidase toxicity test. In order to test the normalization procedure, EC₅₀ values were obtained after short-term exposure to Pb of several dilutions of the same biofilm suspension and the experiment was repeated with three different biofilm samples. EC₅₀ values were expected to increase with TSS concentrations of biofilm suspensions used for metal toxicity tests thus confirming that TSS-normalized EC₅₀ values provide a reliable measure of community tolerance. Second, we wanted to confirm those results by investigating the impacts of Pb exposure in terms of modifications of Pb tolerance after a three-week exposure to Pb in a microcosm experiment. Modifications of both Zn and Cu tolerance and of bacterial and algal community structures (using Automated Ribosomal Intergenic Spacer Analysis or ARISA) were also investigated.

Materials and Methods

Experiment design

Two separate experiments were performed on freshwater biofilms. In experiment 1, natural freshwater biofilms were collected *in situ* and we investigated the influence of biofilm

108 suspensions' concentrations on Pb tolerance measurements using the β -glucosidase toxicity test.
109 In experiment 2, periphyton was grown and exposed to Pb under controlled conditions to assess
110 the impacts of Pb exposure in terms of tolerance acquisition and modifications of community
111 structure.

112

Experiment 1: TSS normalization of Pb EC₅₀ values

Biofilm sampling

Natural freshwater biofilms were collected on three sampling sites located in the vicinity of Paris (North of France): site 1 (Saint-Maurice) is located in the Marne river upstream from Paris (Strahler: 6, mean annual flow: 110 m³/s), site 2 (Bougival) is located in the Seine river downstream from Paris (Strahler: 7, mean annual flow 180 m³/s) and site 3 (Saint-Cyr-sous-Dourdan) is located in the Rémarde river (Strahler: 3, mean annual flow: 0.6 m³/s). Those three sites were chosen to provide three different environments for biofilm growth. Metallic concentrations were not monitored during experiment 1, yet recent contamination data from those 3 sites are provided elsewhere (Fechner et al., 2011; Fechner et al., 2012a; Fechner et al., 2012b) showing that site 2 is more contaminated than sites 1 and 3.

Biofilms were grown on Low Density PolyEthylene (LDPE) membranes (30×10 cm²) vertically attached to plastic crates immersed in the river at the three sampling sites as in Fechner et al. (2010a). Biofilms were collected at each site on different dates after several weeks of *in situ* colonization (> 4 weeks). 3 to 6 colonized membranes were collected at each site and carried back to the laboratory in 250 mL glass bottles filled with mineral water (Grand Barbier, Montdore, France). They were then carefully hand-scraped to remove periphyton. Biofilm suspensions in mineral water were magnetically homogenized and aliquoted for further analyses.

Biofilm parameters

At each sampling date, biofilm dry weights (DWs) were measured in triplicate as in Fechner et al. (2010b). Biofilm suspensions used for the toxicity tests were characterized by their TSS

concentrations expressed in gram of dry matter per liter of biofilm suspension. TSS concentrations were calculated simultaneously with DW concentrations by filtration of a known volume of suspension.

Dilution experiments: influence of the periphyton suspension concentration

For each of the three biofilm samples, four dilutions were prepared in mineral water from a unique biofilm suspension and used to perform four β -glucosidase toxicity tests and determine four EC₅₀ values (see below).

Pb tolerance measurements using the β -glucosidase toxicity test

Pb EC₅₀ values of biofilm samples were obtained using the results from the β -glucosidase toxicity test as in Fechner et al. (2010a). Briefly, biofilms were exposed for one hour to acute concentrations of Pb (six concentrations of metal varying between 0.001 and 10 mM were tested in triplicate for each toxicity test). Pb exposure levels during the toxicity tests were checked by measuring metal concentrations in the stock solutions by flame AAS (Varian Inc., USA). After one hour of exposure, β -glucosidase activity of the metal-exposed biofilms was measured spectrofluorometrically using Methylumbelliferyl- β -D-glucopyranoside (MUF-GLU) (Sigma-Aldrich). Fluorescence of 4-Methylumbelliferone (MUF) was measured using an LB 941 Tristar Ti fluorescence microplate reader (Berthold Technologies, Bad Wildbad, Germany) (excitation/emission filters: 355 and 460 nm). Fluorescence measurements were converted into MUF concentrations by calibrating the spectrofluorimeter with a range of MUF solutions prepared in demineralised water. % inhibition of β -glucosidase activity was then calculated using

MUF concentrations measured for each metal concentration tested. 50% effect concentrations (EC₅₀) and maximum inhibition levels were determined from the dose/effect curves, which were obtained by plotting the % inhibition of β-glucosidase activity as a function of metal concentration, and by fitting the data to the Hill model as described elsewhere (Fechner *et al.*, 2010a). The general form of the curve fitted on the data is:

$$F(x) = \%max \times \frac{x^H}{x^H + EC_{50}^H}$$

where x is the metal concentration, %max is the predicted effect when the toxicant concentration is infinite, and H is the Hill number (slope of the toxicity curve). Each parameter (%max, EC₅₀ and H) was adjusted for the calculation by non-linear regression. 95% confidence intervals around the fitted parameters were estimated using a Bootstrap method. Parameters (EC₅₀ values and maximum % inhibition) were considered significantly different when 95% confidence intervals were not overlapping.

TSS-normalized EC₅₀ values were calculated by dividing each EC₅₀ value by the TSS concentration of the biofilm suspension used for Pb toxicity testing. Data from the four toxicity tests were also gathered on the same plot to produce a unique dose/effect curve with the % inhibition of β-glucosidase activity as a function of TSS-normalized metal concentrations from the four toxicity tests. A unique normalized EC₅₀ value was also calculated from this dose/effect curve for each biofilm sample.

Experiment 2: long-term exposure to Pb in microcosms

Biofilm exposure to Pb in microcosms

A biofilm suspension obtained from site 3 was used to inoculate microcosms (10L plastic tanks) at the beginning of experiment 2. Site 3 was chosen because it is relatively unaffected by the urban area of Paris in terms of metallic contamination (Fechner *et al.*, 2011).

The medium used to cultivate biofilm in the microcosms was mineral water (Aquarel, Nestlé Waters, France) enriched with nutrients (N, P, Si) so as to reach concentrations of 1 mg/L PO_4^{3-} , 20 mg/L NO_3^- and 32 mg/L SiO_2 , which were close to nutrient concentrations measured at site 3/ Aquarel mineral water was chosen for the medium so that physico-chemical parameters were roughly similar to those from the river at site 3 (Fechner *et al.*, 2011).

In each microcosm, periphyton grew on clean immersed LDPE membranes (140 cm², 14 membranes per aquarium) at 20°C, under continuous water mixing conditions using water pumps (Eheim Compact 300, 300 L/h) and a light regime of 13h/11h light/dark cycle using neon lamps (Sylvania, Gro-Lux, F14W-GRO-T8) during three weeks. The medium was changed weekly during the three weeks of colonization. Prior to the beginning of experiment 2, indoor plastic aquaria and LDPE membranes were rinsed with 10% HNO_3 , then twice with distilled water, and finally left to equilibrate for 24h with the cultivation medium spiked with Pb to limit adsorption of the metal on the containers' walls.

Cultivation medium was spiked with stock solutions of PbNO_3 to expose biofilm to three Pb concentrations (1, 10 and 100 µg Pb/L nominal concentrations). One replicate microcosm was used per treatment and a fourth microcosm was used as control (aquaria filled with metal-free medium), with one replicate microcosm per treatment. Pb concentrations in the aquaria were measured daily in aliquots of 5 mL of water: the decrease in metal concentration, mainly due to

metal absorption by biofilm, was corrected by adding stock solutions of PbNO_3 as required ($\leq 100 \mu\text{L}$ per aquarium).

Exposure concentrations were chosen so as to remain realistic from an environmental point of view for the study of aquatic systems under diffuse urban pressure such as the Seine river in the area of Paris, where total Pb concentrations reaching $5 \mu\text{g/L}$ have been recorded (Fechner et al., 2012a).

Laboratory analyses

Water samples were collected daily from the aquaria for measurement of physico-chemical parameters (major anions and cations) and metal concentrations. Pb concentrations were measured daily on 5-mL samples acidified to 1% HNO_3 (suprapure, Merck) by graphite furnace Atomic Absorption Spectrometry (AAS). Validity of the method was checked daily with a certified reference material (EP-L-2, SCP Science).

Biofilm parameters

After three-weeks of biofilm growth, the colonized membranes from each microcosm were scraped in a known volume of mineral water (Grand Barbier, Montdore, France) as in Fechner et al. (2010a) to form a single biofilm sample per aquarium which was homogenized by magnetic stirring. Biofilm samples are hereafter referred to as Pb 0 (control), Pb 1, Pb 10 and Pb 100 according to Pb exposure levels. Aliquots of biofilm suspensions were assigned to various analyses in terms of biomass (DW and Ash-Free Dry Weight or AFDW), metal accumulation and tolerance acquisition were determined as in Fechner et al. (2011). To perform ARISA, three

221 aliquots of 50 mL of each biofilm suspension were centrifuged for 15 minutes at 10 000 g, and
222 4°C. Supernatants were discarded and pellets were frozen and stored at -80°C for further use.

223 AFDW and Pb accumulation in biofilms were performed as in Fechner et al. (2011). Intracellular
224 or non-exchangeable metal concentrations were estimated after washing biofilm samples with
225 EDTA at 4 mM for 10 min according to Meylan et al. (2004).

226
227 *Tolerance measurements with β -glucosidase toxicity test*

228 Pb tolerance levels of Pb 0, Pb 1, Pb 10 and Pb 100 biofilms were assessed by the means of the β -
229 glucosidase toxicity test as in experiment 1. Community tolerance to metals was assessed by
230 calculation of normalized EC₅₀ values (by dividing each EC₅₀ value by the TSS concentration of
231 the periphyton suspension used for the short-term test). Cu and Zn tolerance levels of both Pb 0
232 and Pb 100 biofilms were also measured using the β -glucosidase toxicity test and by calculating
233 TSS-normalized Cu and Zn EC₅₀ values.

235 *ARISA fingerprinting of periphyton*

236 DNA extraction, PCR-amplification and ARISA was performed on triplicates of biofilm pellets
237 as in Fechner et al. (2011). Briefly, primers ITSF/ITSReub (Cardinale *et al.*, 2004) were used for
238 amplification of bacterial 16S-23S intergenic spacers and primers 2234C/3126T (Ranjard *et al.*,
239 2001) were used for amplification of eukaryotic ITS1-5.8S-ITS2 regions. PCR-amplified
240 fragments were separated on an electrophoresis Bioanalyzer (2100 Electrophoresis Bioanalyzer,
241 Agilent Technologies, Santa Clara, CA) and fluorescence data was converted into
242 electrophoregrams using 2100 Expert software (Agilent Technologies, Santa Clara, CA, US).

243 Comparisons between ARISA fingerprints (either bacterial or eukaryotic) were made using the
244 Jaccard index as a % of similarity between two fingerprints from the same experiment using the
245 2100 Expert software (Agilent Technologies, Santa Clara, CA, US): $D(i,j) = a/(a+b+c)$, where a =
246 number of fragments contained in both samples, b = number of fragments contained in sample i
247 but not j, c = number of fragments contained in sample j but not i. This allows comparison
248 between fingerprints using solely the presence or absence of fluorescence peaks and without
249 taking into account peak intensity. To include the maximum number of peaks while excluding
250 background fluorescence, only peaks with a fluorescence value superior to 20 FU (Fluorescence
251 Units) were considered for calculation of the Jaccard indexes. ARISA fragment lengths differing
252 by more than 5 bp were considered distinct.

253 Electrophoregrams in ASCII formats were also imported into the StatFingerprints R package
254 (Michelland *et al.*, 2009) using the 2.10.1 version of the R project. ARISA profiles were aligned
255 and the area under each curve was normalized to 1 before calculation of Shannon indexes, to

assess community diversity using the area under peaks. For calculation of Shannon indexes, all fluorescence peaks were considered (no fluorescence threshold).

Results and discussion

Experiment 1: normalization of Pb tolerance measurements on freshwater biofilms

Our previous study (Fechner et al., 2010a) showed that EC₅₀ values obtained on a given biofilm sample vary with the concentration of the biofilm suspension used for metal toxicity testing. Yet, using the PICT approach requires reliable comparisons of tolerance levels. It is therefore important to develop a measure of community tolerance that does not depend on experimental parameters like the concentration of the suspension used for metal toxicity testing. Our results show that, for Pb also, the higher the TSS concentration of the biofilm suspension, the higher the EC₅₀ value obtained (Figure 1 and Table 1). As observed previously with Cd, Cu, Ni and Zn, mineral or organic particles from the biofilm suspension seem to affect Pb bioavailability during the short-term test. Therefore, when the suspension has a high TSS concentration, Pb bioavailability is smaller and the EC₅₀ value obtained is higher. TSS concentration provides a rough estimate of mineral or organic matter that modifies metal bioavailability during short-term metallic exposure without presuming on the nature of the matter considered, which could be either organic or mineral particles from the river, EPS from the biofilm matrix, etc. This tendency was observed with three different biofilm samples which validates the fact that the influence of the biofilm suspension's concentration on Pb tolerance measurement is not related to the particular composition of a biofilm sample but to modifications of metal bioavailability during the toxicity test.

Defining a TSS-normalized EC_{50} value as a measure of biofilm tolerance therefore appears as a reliable means to reduce the variability of tolerance measurements due to experimental factors. Indeed, the TSS concentration of the biofilm suspension used for metal toxicity testing is related to the number and size of colonized membranes that were collected to sample periphyton, the volume of mineral water used to prepare the suspension, and mostly biofilm growth in the river, which cannot be controlled. Figure 1 shows how TSS-normalization enables to merge data from four toxicity tests performed on four dilutions of the same biofilm sample (left graphs) into a unique dose/effect curve (right graphs) simply by representing the inhibition percentage of β -glucosidase activity as a function of TSS-normalized Pb concentrations (right graphs) instead of Pb exposure concentrations (left graphs).

With TSS-normalization, the variability of Pb tolerance measurements with the concentration of the biofilm suspension used for metal toxicity testing is considerably reduced (Table 1). Results from experiment 1 confirm that Pb normalized EC_{50} value are robust and reliable measures of river biofilm tolerance to Pb.

Experiment 2: impact of Pb exposure on biofilm tolerance and community structure

Experiment 2 aimed at assessing the impacts of Pb on freshwater biofilm after a long-term exposure in microcosms using the β -glucosidase toxicity test. Pb concentrations measured in the exposed biofilms confirm that they accumulated Pb during exposure (Table 2). Yet Pb accumulation was much smaller than previously observed for metals like Cd, Ni and Zn (Fechner et al., 2011). For instance, the bioconcentration factor (total biofilm-accumulated metal in $\mu\text{g/g}$ DW divided by the nominal metal concentration in the corresponding microcosm in $\mu\text{g/L}$) varied

from 0.50 (Pb 1) to 0.06 (Pb 10 and Pb100) whereas in our previous study the bioconcentration factor reached 11 and 40 for respectively Cd and Zn-exposed biofilms (Fechner et al., 2011). Moreover, Pb accumulation was not significant for biofilms exposed to 1 and 10 µg/L Pb (Table 2).

Pb exposure was accompanied by modifications of community structures as revealed by ARISA for both bacterial and eukaryotic communities. Indeed both the Shannon index and the number of fluorescence peaks tended to decrease for Pb-exposed biofilms especially for the Pb 100 biofilm (Table 3). Moreover, bacterial community structure was influenced by metal exposure as Pb 0 (control) and Pb 100 (highest exposure level) biofilms had the lowest percentage of similarity (Jaccard index, Table 4) whereas Pb 10 and Pb 100, which correspond to the two highest exposure levels, shared the highest percentage of similarity. As far as eukaryotic communities are concerned, the percentage of similarity was surprisingly high for Pb 0 and Pb100 biofilms but was also high for Pb 10 and Pb 100 biofilms. Exposure to Pb also induced a decrease of β -glucosidase activity especially for the Pb 100 biofilm (Table 2) which indicates a functional impact of Pb exposure at 100 µg/L Pb. DW and AFDW also decreased for the Pb 100 biofilm but increased slightly for Pb 1 and Pb 10 communities. A similar effect was observed on DW for biofilms exposed to low metal concentrations (Morin et al., 2008; Fechner et al., 2011).

The β -glucosidase toxicity test proved to be an efficient tool to detect Pb-induced tolerance. Two biological responses can be derived from the toxicity test: a measure of community tolerance (normalized EC₅₀ value) and a maximum % inhibition of β -glucosidase activity (Table 5). A maximum % inhibition inferior to 100% means that β -glucosidase activity cannot be fully inhibited by short-term exposure to metals even at high exposure concentrations (the highest metal concentrations tested are 10 mM namely 2.07 g/L for Pb). This phenomenon was already

observed in previous studies on laboratory or *in situ* exposed biofilms with the same toxicity test (Fechner et al., 2011; Fechner et al., 2012a). In the present study, β -glucosidase toxicity tests revealed the impact of Pb exposure on the Pb 100 biofilm with a 1.7 increase of normalized EC_{50} value and a significant decrease of maximum % inhibition (72.30 %) when compared to Pb 0 (Figure 2 and Table 5).

The maximum inhibition % remained close to 100 % for Pb 0, Pb 1 and Pb 10 biofilms and no increase of the normalized EC_{50} value was observed after exposure to 1 and 10 $\mu\text{g/L}$ Pb (Table 5). A similar phenomenon was observed for Cd, Ni and Zn in our previous study: tolerance acquisition to each metal could only be observed for high exposure levels: 3 $\mu\text{g/L}$ Cd, 50 $\mu\text{g/L}$ Ni, 200 $\mu\text{g/L}$ Zn but not for low exposure levels (0.3, 5 and 20 $\mu\text{g/L}$ respectively) (Fechner et al., 2011). Yet those metallic exposure levels remain environmentally-realistic and much smaller than those usually tested in the scientific literature as discussed previously (Fechner et al., 2011). Moreover, those results do not question the sensitivity of tolerance measurement to assess the effects of lower metallic exposures. Indeed, metal tolerance acquisition was already observed in the field at diffuse concentration levels using the same methodology (Fechner et al., 2012a). Therefore, experiment 2 validates the use of the β -glucosidase toxicity test to assess Pb tolerance acquisition at environmental metal concentrations.

Cu and Zn tolerance levels were measured on Pb 0 and Pb 100 biofilms to investigate co-tolerance (Table 5 and Figure 3). The Pb 100 biofilm became tolerant to Zn (3.3 increase of the normalized EC_{50} value when compared to the Pb 0 biofilm and a decrease of the maximum % inhibition to 88.3 %). As far as Cu is concerned, no modification of the normalized EC_{50} value was observed but the % maximum inhibition decreased to 78.3 after short-term exposure to Cu. Those results indicate Pb-induced modifications in the response of the heterotrophic community

to Cu. Co-tolerance is a common phenomenon for algal and bacterial microorganisms and has been previously observed on biofilms (for instance Soldo and Behra, 2000; Fechner et al., 2011) . It can be related to various defense mechanisms either specific (for instance metal efflux or decrease of metal uptake by modification of channel or carrier proteins used by the metal ions to enter the cells (Harrison et al., 2007) or non-specific, for instance production of metal-binding EPS (Mason and Jenkins, 1995; Jang et al., 2001) to a metal. The fact that exposure to Pb induced tolerance to both Pb and Zn but not to Cu suggests that, in this case, the exposed community probably developed defense mechanisms that are specific to Pb and Zn. Some of the mechanisms that are described in the literature might explain co-tolerance to both Pb and Zn: Zn-CPx-type ATPases which transport Zn^{2+} , Cd^{2+} and Pb^{2+} from the cytoplasm to the outside of the cell or to the periplasm could be involved (Rensing et al., 1999; Nies, 2003). For instance, PbrA, one of the Zn-CPx-type ATPases, was shown to be part of a plasmid-mediated Pb resistance determinant in *R. metallidurans* (Nies, 2003). Co-tolerance to Zn had previously been described using the same methodology for tolerance measurements after exposure to Cd (Fechner et al., 2011). Co-tolerance has implications for use of tolerance measurements to assess the impacts of toxic exposure in the field. Indeed, it means that proper identification of the exact selection pressure is difficult. In the present study, tolerance to Zn is induced by exposure to Pb and not by exposure to Zn. In field studies in a context of urban multi-contamination, tolerance to Zn can therefore not be directly linked to Zn exposure levels. Co-tolerance mechanisms are numerous and multiple patterns of co-tolerance might be observed depending on exposure concentrations and biofilm composition. It is thus probable that metal tolerance has to be considered as an early-warning response to a mixture of metals but cannot be used to investigate exposure to a specific metal.

369

370 **Conclusion**

371 This study focused on the assessment of robust Pb tolerance measurements on freshwater
372 biofilms. Our results show that the methodology previously developed (tolerance measurement
373 with the β -glucosidase toxicity test and TSS-normalization of EC_{50} value) can be used to measure
374 Pb tolerance. Experiment 1 confirms that TSS-normalized EC_{50} values can be reliably used to
375 compare Pb tolerance levels of different biofilm samples. Moreover, the methodology allowed
376 the detection of tolerance acquisition after long-term exposure to Pb under controlled conditions
377 (experiment 2). Co-tolerance to Zn was observed for the biofilm exposed to 100 $\mu\text{g/L}$ Pb, but no
378 modification of Cu tolerance was observed. Tolerance acquisition was accompanied by
379 modifications of community structure as revealed by fingerprinting and by the appearance of
380 maximum % inhibitions of β -glucosidase activity inferior to 100%. This parameter appears as an
381 interesting biological response obtained with the β -glucosidase toxicity test. It was indeed
382 observed after short-term exposure to Cu for the biofilm exposed to 100 $\mu\text{g/L}$, whereas no
383 increase of the EC_{50} value was detected. This study shows that the impacts of Pb exposure can be
384 detected after exposure to environmental concentrations of Pb using a PICT approach.

385

386 **Acknowledgements**

387 This study was supported by ONEMA (Office National de l'Eau et des Milieux Aquatiques). We
388 are grateful to Aurélie Germain for her valuable help in the laboratory and to Julien Guieu for
389 editing the English version of the manuscript.

390 **Table 1.** EC₅₀ and normalized EC₅₀ (with their 95% confidence intervals) obtained during
391 experiment 1 on biofilms collected at sites 1, 2 and 3. EC₅₀ values are expressed in mgPb/L and
392 normalized EC₅₀ values in en mgPb/gTSS.

393

Sampling site	TSS concentration (standard deviation) of each biofilm suspension	EC ₅₀ (and 95% confidence intervals)	Normalized EC ₅₀ (and 95% confidence intervals)	Global normalized EC ₅₀ from the unique dose/effect curve
Site 1 (Marne river)	17.63 (0.14) 5.98 (1.08) 2.78 (0.41) 1.03 (0.10)	525.01 (434.52 - 651.51) 177.64 (159.59 - 201.23) 116.16 (101.38 - 140.43) 70.98 (53.41 - 86.80)	29.79 (22.59 - 36.98) 29.70 (18.48 - 40.93) 41.76 (28.55 - 54.98) 68.91 (47.76 - 90.06)	39.32 (34.41 - 46.98)
Site 2 (Seine river)	13.94 (0.49) 2.84 (0.06) 1.24 (0.10) 0.49 (0.05)	329.00 (192.34 - 456.88) 70.86 (62.43 - 79.35) 47.72 (38.53 - 57.74) 21.59 (17.44 - 26.11)	23.60 (13.66 - 33.54) 24.98 (21.79 - 28.17) 38.55 (28.41 - 48.69) 43.61 (30.53 - 56.69)	33.54 (28.73 - 38.76)
Site 3 (Rémarde river)	1.75 (0.17) 1.27 (0.45) 0.90 (0.01) 0.34 (0.07)	149.92 (136.53 - 166.88) 100.26 (93.43 - 106.49) 59.67 (47.15 - 86.42) 34.25 (28.53 - 48.58)	85.48 (66.91 - 104.05) 79.26 (24.29 - 134.23) 66.06 (36.38 - 95.74) 99.75 (42.42 - 157.08)	85.36 (79.45 - 91.87)

394

Table 2. Impact of Pb on biofilm parameters: DW, AFDW (both expressed in μg dry matter/ cm^2 of colonized membrane) and β -glucosidase activity (expressed in μM MUF/g of organic matter/h) and on biofilm-accumulated Pb concentrations (total and intracellular) expressed in $\mu\text{gPb/g}$ of dry matter (DW). Statistical differences were assessed using a non-parametric test (Kruskal-Wallis followed by the Dunn test for pairwise comparisons of means) as few replicates were measured (n= 3). Different letters indicate significant differences between biofilm samples (p = 0.05). No statistical differences was be assessed for biofilm-accumulated metals as n = 2.

metal treatment	biofilm parameters			biofilm-accumulated metals	
	DW	AFDW	β -glucosidase activity	total	intracellular
Pb 0	179.37 ^{ab} (22.50)	75.40 ^{ab} (0.75)	169.60 ^a (0.73)	0.56	0.70
Pb1	287.30 ^{ab} (24.09)	95.24 ^{ab} (0.95)	92.98 ^{ab} (5.50)	0.50	0.52
Pb 10	461.31 ^a (16.57)	150.99 ^a (1.51)	151.36 ^{ab} (2.83)	0.59	0.49
Pb 100	150.79 ^b (10.41)	44.97 ^b (0.45)	20.18 ^b (0.30)	6.47	4.26

Table 3. Shannon-Weaver index and number of major fluorescence peaks (fluorescence > 20 FU) from ARISA profiles of Pb 0, 1, 10 and 100 biofilms from experiment 2. Standard deviations are shown in brackets (n = 3).

Pb exposure levels	Bacteria		Eukaryotes	
	Shannon index	Number of peaks	Shannon index	Number of peaks
Pb 0	2.14 (0.15)	8	1.78 (0.14)	9
Pb 1	2.14 (0.34)	8	1.19 (0.004)	7
Pb 10	2.02 (0.4)	5	1.55 (0.05)	7
Pb 100	1.5 (0.03)	4	1.34 (0.06)	8

413 **Table 4.** % of similarity between (bacterial and eukaryotic) ARISA profiles corresponding to the
414 four exposure concentrations (experiment 2).

415

% of similarity	Bacteria	Eukaryotes
Pb 0/Pb 1	28.57%	14.29%
Pb 0/Pb 10	27.27%	40.00%
Pb 0/Pb 100	18.18%	45.45%
Pb 1/Pb 10	33.33%	18.18%
Pb 1/Pb 100	25.00%	16.67%
Pb 10/Pb 100	37.50%	44.44%

416 **Table 5.** Normalized EC₅₀ values and maximum inhibition levels of β-glucosidase activity
 417 calculated from the dose/effect curves. with their 95% confidence intervals (experiment 2).

Pb exposure levels	Short-term toxicity tests	normalized EC ₅₀ in mg _{metal} /g _{TSS} (95% confidence interval)	% maximum inhibition level (95% confidence interval)
Pb 0	Pb	15.81 (13.18-18.44)	98.92 (98.01-99.78)
Pb 1		17.73 (7.61-27.86)	92.38 (90.85-93.65)
Pb 10		15.76 (13.62-17.90)	99.01 (97.71-100)
Pb 100		26.64 (16.40-36.87)	72.30 (68.92-78.18)
Pb 0	Cu	1.37 (0.88-1.86)	100 (98.56-100)
Pb 100		1.34 (1.09-1.60)	78.29 (74.37-82.22)
Pb 0	Zn	1.79 (1.36-2.22)	99.69 (98.59-100)
Pb 100		4.51 (3.87-5.14)	88.24 (87.03-89.45)

418

Figure captions

Fig. 1 Dose/effect curves from experiment 1 showing % inhibition of β -glucosidase activity as a function of Pb exposure concentrations expressed in mg/L (left graphs) and of TSS-normalized Pb concentrations (right graphs) expressed in gPb/gTSS for each biofilm sample (1: site 1 biofilm sample. 2: site 2 biofilm sample. 3: site 3 biofilm sample)

Fig. 2 Dose/effect curves of biofilms exposed to 0, 1, 10 and 100 μ g/L Pb for three weeks obtained by short-term toxicity tests with Pb (experiment 2). Curves represent the % inhibition of β -glucosidase activity for each Pb concentration tested as a function of TSS-normalized Pb concentrations (expressed in gPb/gTSS)

Fig. 3 Dose/effect curves of Pb 0 and Pb 100 biofilms obtained by short-term toxicity tests with Cu (a) and Zn (b). Curves represent the % inhibition of β -glucosidase activity for each metal concentration tested as a function of TSS-normalized Pb concentrations (expressed in gPb/gTSS)

434 **References**

- 435 Blanck H (2002) A Critical Review of Procedures and Approaches Used for Assessing Pollution-
436 Induced Community Tolerance (PICT) in Biotic Communities. *Human and Ecological Risk*
437 *Assessment* 8:1003-1034.
- 438 Cardinale M, L Brusetti, P Quatrini, S Borin, AM Puglia, A Rizzi, E Zanardini, C Sorlini, C
439 Corselli, D Daffonchio (2004) Comparison of Different Primer Sets for Use in Automated
440 Ribosomal Intergenic Spacer Analysis of Complex Bacterial Communities. *Appl. Environ.*
441 *Microbiol.* 70(10):6147-6156.
- 442 Fechner LC, C Gourlay-Francé, A Bourgeault, MH Tusseau-Vuillemin (2012a) Diffuse urban
443 pollution increases metal tolerance of natural heterotrophic biofilms. *Environmental Pollution*
444 162:311-318.
- 445 Fechner LC, C Gourlay-Francé, MH Tusseau-Vuillemin (2011) Low exposure levels of urban
446 metals induce heterotrophic community tolerance: a microcosm validation. *Ecotoxicology*
447 20(7):793-802.
- 448 Fechner LC, C Gourlay-Francé, E Uher, MH Tusseau-Vuillemin (2010a) Adapting an enzymatic
449 toxicity test to allow comparative evaluation of natural freshwater biofilms' tolerance to metals.
450 *Ecotoxicology* 19(7):1302-1311.
- 451 Fechner LC, F Versace, C Gourlay-Francé, MH Tusseau-Vuillemin (2012b) Adaptation of
452 copper community tolerance levels after biofilm transplantation in an urban river. *Aquatic*
453 *Toxicology* 106-107:32-41.
- 454 Fechner LC, F Vincent-Hubert, P Gaubert, T Bouchez, C Gourlay-Francé, MH Tusseau-
455 Vuillemin (2010b) Combined eukaryotic and bacterial community fingerprinting of natural
456 freshwater biofilms using Automated Ribosomal Intergenic Spacer Analysis. *Fems Microbiology*
457 *Ecology* 74(3):542-553.
- 458 Harrison JJ, H Ceri, RJ Turner (2007) Multimetal resistance and tolerance in microbial biofilms.
459 *Nature Reviews Microbiology* 5(12):928-938.
- 460 Jang A, SM Kim, SY Kim, SG Lee, IS Kim (2001) Effects of heavy metals (Cu, Pb, and Ni) on
461 the compositions of EPS in biofilms. *Water Science and Technology* 43(6):41-48.
- 462 Lamelas C, JP Pinheiro, VI Slaveykova (2009) Effect of humic acid on Cd(II), Cu(II), and Pb(II)
463 uptake by freshwater algae: Kinetic and cell wall speciation considerations. *Environmental*
464 *Science and Technology* 43(3):730-735.
- 465 Mason AZ, KD Jenkins (1995) Metal Detoxification in Aquatic Organisms. In: Tessier A, Turner
466 DR (eds) *Metal Speciation and Bioavailability in Aquatic Systems*. John Wiley & Sons Ltd, New
467 York, NY, USA.
- 468 Meylan S, R Behra, L Sigg (2004) Influence of Metal Speciation in Natural Freshwater on
469 Bioaccumulation of Copper and Zinc in Periphyton: A Microcosm Study. *Environ. Sci. Technol.*
470 38(11):3104-3111.
- 471 Michelland RJ, S Dejean, S Combes, L Fortun-Lamothe, L Cauquil (2009) StatFingerprints: a
472 friendly graphical interface program for processing and analysis of microbial fingerprint profiles.
473 *Molecular Ecology Resources* 9(5):1359-1363.
- 474 Morin S, T Duong, O Herlory, A Feurtet-Mazel, M Coste (2008) Cadmium Toxicity and
475 Bioaccumulation in Freshwater Biofilms. *Archives of Environmental Contamination and*
476 *Toxicology* 54(2):173-186.
- 477 Nies DH (2003) Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiology*
478 *Reviews* 27(2-3):313-339.

Pesce S, C Margoum, B Montuelle (2010) In situ relationships between spatio-temporal variations in diuron concentrations and phototrophic biofilm tolerance in a contaminated river. *Water Research* 44(6):1941-1949.
 Ranjard L, F Poly, JC Lata, C Mougél, J Thioulouse, S Nazaret (2001) Characterization of Bacterial and Fungal Soil Communities by Automated Ribosomal Intergenic Spacer Analysis Fingerprints: Biological and Methodological Variability. *Appl. Environ. Microbiol.* 67(10):4479-4487.
 Rensing C, M Ghosh, BP Rosen (1999) Families of soft-metal-ion-transporting ATPases. *Journal of Bacteriology* 181(19):5891-5897.
 Sabater S, H Guasch, M Ricart, A Romaní, G Vidal, C Klünder, M Schmitt-Jansen (2007) Monitoring the effect of chemicals on biological communities. The biofilm as an interface. *Analytical and Bioanalytical Chemistry* 387(4):1425-1434.
 Sabater S, H Guasch, A Romaní, I Muñoz (2002) The effect of biological factors on the efficiency of river biofilms in improving water quality. *Hydrobiologia* 469(1):149-156.
 Sánchez-Marín P, J Santos-Echeandía, M Nieto-Cid, XA Álvarez-Salgado, R Beiras (2010) Effect of dissolved organic matter (DOM) of contrasting origins on Cu and Pb speciation and toxicity to *Paracentrotus lividus* larvae. *Aquatic Toxicology* 96(2):90-102.
 Soldo D, R Behra (2000) Long-term effects of copper on the structure of freshwater periphyton communities and their tolerance to copper, zinc, nickel and silver. *Aquatic Toxicology* 47(3-4):181-189.
 Tsiridis V, M Petala, P Samaras, S Hadjispyrou, G Sakellariopoulos, A Kungolos (2006) Interactive toxic effects of heavy metals and humic acids on *Vibrio fischeri*. *Ecotoxicology and Environmental Safety* 63(1):158-167.